MITOGEN-ACTIVATED PROTEIN KINASE AND METHOD OF USE TO ENHANCE BIOTIC AND ABIOTIC STRESS TOLERANCE IN PLANTS

1. REELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application Serial No. 60/444,249 filed January 31, 2003, which is incorporated by reference herein.

2. FIELD OF INVENTION

The present invention relates to an abscisic acid-inducible mitogen-activated protein kinase (MAPK) and the use of MAPK for increasing abiotic stress tolerance and disease resistance in monocots.

3. BACKGROUND OF THE INVENTION

Plants are constantly exposed to a variety of biotic stress such as pathogen infection or insect herbivory and abiotic stresses such as high or low temperatures, drought and salinity. To survive these challenges, plants have developed elaborate mechanisms to detect external signals and manifest adaptive responses with proper physiological and morphological changes (Bohnert et al., 1995). Detection of extracellular stimuli and subsequent activation of defense responses requires a complex interplay of signaling cascades in which reversible protein phosphorylation plays a central role (Yang et al., 1997).

Increasing evidence has shown that the intracellular signaling module, the mitogen-activated protein kinase (MAPK) cascade plays an important role in plant signal transduction related to biotic and abiotic stresses. This phosphorylation cascade typically consists of three functionally interlinked protein kinases: a MAP kinase kinase kinase (MAPKKK), a MAP kinase kinase (MAPKK) and a MAP kinase (MAP kinase). In this phosphorylation module, a MAPKKK phosphorylates and activates a particular MAPKK which in turn phosphorylates and activates a MAPK. Activated MAPK is often imported into the nucleus where it phosphorylates and activates specific downstream signaling components such as transcription factors (Khokhlatchev et al., 1998).

Activation of MAPKs has been observed in plants exposed to pathogens (Suzuki and Shinshi, 1995; Adam etal., 1997; Ligternik et al., 1997; Zhang and Klessig, 1997, 1998b; He

et al., 1999), cold (Jonak et al, 11996), and wounding (Seo et al., 1995; Usami et al., 1995; Bogre et al., 1997; Zhang and Klessig, 1998a; Seo et al., 1999; He et al., 1999). Plant MAPKs can also be activated by fungal elicitors (Suzuki and Shinshi, 1995), salicylic acid (Zhang and Klessig, 1997), jasmonic acid (Seo et al., 1999), and abscisic acid (Knetsch et al., 1996; Burnett et al., 2000; Heimorvaara-Dijkstra et al., 2000). Although, considerable progress has been made in cloning and characterization of plant MAPKKs (Morris et al., 1997; Ichimura et al., 1998a; Hackett et al., 1998; Hardin and Wolniak, 1998; Kiegerl et al., 2000 Yang et a., 2001) and MAPKKKs (Ichimura et al., 1998b; Kovtun et al., 2000; Frye et al., 2001), detailed steps of MAP kinase cascades have yet to be elucidated in any plant species. Upstream MAPKKs for dicot MAPKs such as NtMEK2 for SIPK/WIPK in tobacco (Yang et., 2001), AtMEK1 for AtMPK4 in *Arabidopsis* (Huang et al., 2000), and SIMKK for SIMK in alfalfa (Kiegerl et al., 2000) have been determined. The complete MAP kinase cascade (MEKK1), MKK4/MKK5 and MPK3/MPK6 together with its upstream receptor kinase FLS2 and downstream WRKY22/WRKY29 transcription factors was characterized in *Arabidopsis* (Asai et al., 2002). These findings suggest that MAPKs are important signaling components in plant defense responses and that the cascade of a "three-kinase module" is a general mechanism of defense signal transduction among eukaryotic organisms (Ligterink and Hirt, 2000).

Recently, protein kinases possessing close sequence similarity to the mammalian MAPKs have been identified in plants (Stone and Walker, 1995; Hirt, 1997; Mizoguchi et al, 1997; Tena etal., 2001; Ahang and Klessig, 2001; Ichimura et al., 2002). However, despite this progress, most characterized plant MAPKs were isolated from dicot model species such as *Arabidopsis* and tobacco and our understanding of the role of MAPK cascades in stress response remains rather limited. Moreover, very few MAPKs have been identified and characterized in economically important monocot species such as rice, maize, wheat or barley. Rice is not only principal food crop for over half of the world's population, but also an excellent model for cereal crops because of its relatively small genome, extensive genetic mapping data, relatively easy transformation and synteny with other cereal genomes. A MAP kinase, OsBWMK1 found in rice leaf was determined to be activated by blast fungus infection and wounding (He et al., 1999) and a stress-responsive rice MAP kinase gene (variously named *OsMAPK5*, *OsMSRMK2*, *OsMAPK2*, *OsMAP1* or *OsBIMK1*)

was identified and shown to be induced at the mRNA level by multiple biotic and abiotic stresses (Xiong et al., 2001; Agrawal et al., 2002; Huang et al., 2002: Wen et al., 2002; Song et al., 2002). Plant MAPKs are encoded by a multigene family and play a pivot role in plant growth and development as well as biotic and abiotic stress responses. As a result, functional genomic analysis of the entire MAPK gene family in rice should significantly enhanced our understanding of the MAPK-mediated signaling network in monocots and its effects on agronomically important traits such as yield, quality, pest resistance and abiotic stress tolerance.

Discussion or citation of a reference herein shall not be construed as an admission that such reference is prior art to the present invention.

4. SUMMARY OF THE INVENTION

This invention relates to a phosphorylation protein called mitogen-activated protein kinase (MAPK) and its role in mediating stress responses in plants. Specifically, the present inventors discovered that the rice mitogen-activated protein kinase gene called OsMAPK5 is differentially spliced generating at least two genes. Accordingly, the present invention encompasses the nucleotide sequences that encode the OsMAPK5, including mutants, isoforms, recombinants and fusion proteins.

Prior studies suggested that other plant MAPK genes are induced by abiotic stresses including drought, salinity and low temperature (Jonak etl., 1996, Munnik et al., 1999; Mikolajczk et al., 2000; Berberich et al., 1999; Huang et al., 2002; Agrawal et al., 2002). However, none of these studies revealed any functional analysis or regulatory correlations with abiotic stresses. The present inventors demonstrated for the first time that an abscisic acid inducible rice mitogen activated protein kinase is capable of inversely modulating disease resistance and abiotic stress tolerance. First, overexpression of *OsMAPk5* resulted in enhanced plant tolerance to drought, salt and cold stresses. Secondly, suppression of OsMAPK5 reduced abiotic stress tolerance but led to constitutive *PR* gene expression and increased disease resistance.

Therefore, the present invention further provides methods for evaluating tolerance to abiotic stress or resistance to biotic stress in plants. For example, one method provides for evaluating a plant for tolerance to abiotic stress comprising treating a plant with an

abiotic or biotic stress; isolating MAPK5 protein from the plant; detecting for MAPK5 activity; and evaluating the increase or decrease in MAPK5 activity in the plant whereby the increase in MAPK5 activity indicates the plant is tolerant to stress. MAPK5 or its ortholog is isolated by immunoprecipitating the protein with a MAPK5 protein that specifically binds to MAPK5.

This invention also provides methods for enhancing tolerance to abiotic stress or increasing resistance to biotic stress in a plant. These methods include transforming a plant with MAPK5 nucleic acid sequence wherein the MAPK5 protein is expressed in the plant; treating a plant with an abiotic stress; isolating MAPK5 protein from the plant; detecting for MAPK5 activity; and evaluating the increase or decrease in MAPK5 activity in the transformed plant whereby the increase in MAPK5 activity indicates the increase in tolerance to abiotic stress in the transformed plant compared to the wild-type plant The decrease in MAPK5 activity indicates the increase resistance to biotic stress in the transformed plant compared to the wild-type plant.

The present also provides kits for screening plants for susceptibility to biotic stress or tolerance to abiotic stress. The kit includes an isolated nucleic acid probe that comprises a label and a nucleotide sequence that encodes a polypeptide consisting essentially of the amino sequence of MAPK5 or its complement and at least one reagent suitable for detecting the presence of a nucleic acid molecule encoding MAPK5 whereby the changes in polymorphic patterns of MAPK5 indicates the plant is susceptible to biotic stress. Another kit of the present invention provides for detecting a plant for tolerance to abiotic stress comprising an antibody that immunospecifically binds to a MAPK5 polypeptide wherein the antibody is labeled and at least one reagent suitable for detecting the presence of MAPK5 whereby the increase or decrease in MAPK5 activity indicates the plant is tolerant to abiotic stress.

5. DETAILED DESCRIPTION OF THE FIGURES

Figure 1 represents an amino acid sequence comparison of *OsMAPK5a* and *OsMAPK5a* with MAPKs from other higher plants. (A) Alignment of deduced amino acid sequences of *OsMAPK5a* and *OsMAPK5b* with two closely related MAPKs, TaWCK-1 and NtWIPK. Conserved amino acid residues are listed. The 11 subdomains

of the protein kinases are indicated above the sequences by Roman numbers. Threonine (T) and tyrosine (Y), two residues normally phosphorylated for activation of MAP kinases, are marked by *asterisks*. (B) The phylogenetic relationship of *OsMAPK5a* and *OsMAPK5b* with other plant MAPKs.

Figure 2 represents genomic organization, alternative splicing, recombinant proteins and autophosphorylation activity of *OsMAPK5*. (A) Southern blot analysis of the *OsMAPK5* gene. (B) RT-PCR analysis using a primer pair covering the differentiated regions of the *OsMAPK5a* and *OsMAPK5*b cDNAs. Lane 1 shows RT-PCR analysis of two days post infection blast fungus-induced mRNAs from the cultivar Drew. Lanes 2 and 3 represent PCR analysis of *OsMAPK5a* and *OsMAPK5b* cDNAs. (C) *In vitro* expression of *OsMAPK5a* and *OsMAPK5b*, and the specificity of the *OsMAPK5* antibody. One hundred nanograms of the total protein from *E. coli* (left lanes) or 10 ng (right lanes) of affinity-purified fusion protein of His-OsMAPK5a and His-OsMAPK5b were separated on 10% SDS-PAGE and detected with the anti-OsMAPK5 antibody. (D) *In vivo* autophosphorylation assay of affinity-purified fusion proteins, His-OsMAPK5a and His-*OsMAPK5b*.

Figure 3 represents activation of *OsMAPK5*, its protein and kinase activity by inoculation with the blast fungus. Assays were repeated three times using samples from independent experiments. (A) Northern blot analysis of *OsMAPK5* expression using the same gene-specific probe used in Southern blot analysis. Equal loading of total RNAs (20 μg per lane) was verified using rice 28S ribosomal RNA as a loading control. (B) Immunoblot analysis of OsMAPK5. (C) MBP in-gel kinase assay. Only the band corresponding to the activity of OsMAPK5a was shown since no activity was detected for OsMAPK5b.

Figure 4 represents induction of *OsMAPK5*, its protein and kinase activity by ABA and wounding. (A) Northern blot analysis of *OsMAPK5* expression in two-week-old seedlings treated with 0.1 mM ABA, 1mM SA, 0.1 mM JA or wounding. Total RNAs were extracted at the specified time. The same blots were probed with *PBZ1* cDNA. (B)

Immunoblot analysis of *OsMAPK5* in two-week-old seedlings treated with 0.1 mM ABA, 1 mM SA, 0.1 mM JA or wounding. (C) MBP in-gel kinase activity of the immunoprecipitated *OsMAPK5* from two-week-old seedlings treated with 0.1 mM ABA, 1 mM SA, 0.1 mM JA or wounding.

Figure 5 represents induction of *OsMAPK5*, its protein and kinase activity by drought, salt and low temperature. Experiments were repeated three times by using samples from independent treatments. (A) Northern blot analyses of *OsMAPK5* expression in two-week-old seedlings subjected to drought (water withheld up to 5 days); salt (200mM NaCl) or cold (4°C) stress. (B) Immunoblot analyses of *OsMAPK5* under drought (root tissues), salt (root tissues) and cold (leaf tissues) stresses. (C) MBP in-gel kinase activity assay of the immunoprecipitated *OsMAPK5* under drought (root tissues), salt (root tissues) and cold (leaf tissues) stresses.

Figure 6 represents overexpression and suppression of *OsMAPK5* in transgenic rice. (A) The overexpression construct (*OsMAPK5-OX*) under the control of the CaMV 35S promoter introduced into Nipponbare by the *Agrobacterium*-mediated transformation. Thirty independent T_0 transgenic lines were obtained and examined (5 representative lines and control plant, Nipponbare, are shown) for the OsMAPK5 expression and kinase activity under normal growth condition. The base level of endogenous OsMAPK5 in control plants was not detected under the optimal exposure time for detecting the overexpressed OsMAPK5 (see Figures 3 and 4). (B) The double-stranded RNA interference construct (*OsMAPK5-RI*) under the control of the CaMV 35S promoter introduced into Nipponbare by the *Agrobacterium*-mediated transformation. Endogenous OsMAPK5 protein levels and kinase activities in the transgenic lines were examined using rice leaves infected with the fungal isolate IC17-18/1 at 3 days after spot-inoculation. (C) Development of brownish stripes on mature flag leaves of *OsMAPK5-RI* transgenic lines. Top and bottom represent control and transgenic rice leaves before and after the removal of chlorophyll (overnight soaking in 100% ethanol), respectively.

Figure 7 represents resistance of *OsMAPK5-RI* lines to the blast fungus. **(A)** Blast resistance evaluation of T_0 trangenic plants by the spot inoculation method of typical disease symptoms on leaves of control plants and overexpression (OX1) and dsRNAi (RII) transgenic plants at 6 days after inoculation with fungal isolate IC17-18/1. **(B)** Blast resistance evaluation of two-week-old T_1 transgenic plants based on disease rating using 20-40 hygromycin-resistant transgenic seedlings per line from three oeverexpression lines, four dsRNAi lines and control line were spray-inoculated with fungal isolate IC-18/1. Disease ratings were performed according to Marchetti's scale (Marchetti et al., 1976) at five days post-inoculation. **(C)** Blast resistance evaluation of T_1 transgenic plants based on lesion numbers per infected leaf at five days post-inoculation. **(D)** Blast resistance evaluation of T_1 transgenic plants based on relative fungal growth. Total RNA from infected leaves at five days post-inoculation was blotted and hybridized with *P. grisea* 28S rDNA and rice 25S rDNA respectively. The fungal 28S *rDNA* hybridization signals were quantified by Phosphoimager and calibrated with rice 25S rDNA signal for equal loading. **(E)** MBP in-gel kinase assay of the immunoprecipated *OsMAPK5* from leaf tissues of control and transgenic lines at five days post-inoculation.

Figure 8 represents *OsMAPK5-RI* lines resistance to bacterial pathogen, *B. glumae*. Leaf sheaths from one-month-old control and T_1 transgenic seedlings were inoculated with *B. glumae* (1×10^6 cfu). At least 10 hygromycin-positive transgenic seedlings per line were used in each experiment. **(A)** Disease resistance evaluation based on lesion size at 7 days post-inoculation. **(B)** Disease resistance evaluation based on the bacterial growth in *planta* at 7 days post-inoculation. **(C)** MBP in-gel kinase assay of immunoprecipitated *OsMAPK5* from leaf tissues at 7 days post-inoculation.

Figure 9 shows constitutive expression of *PR-1* and *PR-10* genes in *OsMAPK5-RI* trangenic lines. Total RNA was isolated form two-week-old control and T_1 transgenic seedlings grown under the normal conditions. The Northern blot was probed sequentially with the *PR-1b*, *PR-10* and rice 25S rDNA with 10 μg of RNA loaded per lane.

Figure 10 shows tolerance of *OsMAPK5-OX* and *OsMAPK-RI* transgenic plants to cold, salt and drought treatments. **(A)** The percentage of survived seedlings after cold treatment at 4°C for 3 days followed by normal growth condition for recovery; salt treatment at 200 mM NaCl for a maximum of 4 days; or drought treatment by withholding water for a maximum of 6 days. At least 40 hygromycin-positive T_1 transgenic seedlings were used in each experiment and repeated twice. Statistical analysis (t-test) was performed to evaluate the levels of cold, salt and drought tolerance based on the percentage of survived seedlings in the overexpression or suppression lines vsersus the control line after the abiotic treatments. **(B)** MBP in-gel kinase assay of immunoprecipitated *OsMAPK5* from mixed leaf tissues samplings at different times under cold treatment at 6, 12, 24 hours; salinity treatment for 6, 12 and 24 hours or drought treatment for 2, 3, 4 days. The relative MBP kinase activities of control and transgenic lines were calculated based on phosphoimaging quantification of the band intensity.

Figure 11 represents the nucleic acid sequence of *OsMAPKa* denoted as SEQ. ID. NO:1.

Figure 12 represents the amino acid sequence of OsMAPKa denoted as SEQ. ID. NO:2.

Figure 13 represents the nucleic acid sequence of *OsMAPKb* denoted as SEQ. ID. NO:3.

Figure 14 represents the amino acid sequence of OsMAPKb denoted as SEQ. ID. NO:4.

6. DETAILED DESCRIPTION OF THE INVENTION

This section presents a detailed description of the invention and its applications. This description is by way of several exemplary illustrations, in increasing detail and

specificity, of the general methods of this invention. These examples are non-limiting, and related variants will be apparent to one of skill in the art.

It should be understood that various changes and modifications to the presently preferred embodiments described herein will be apparent to those skilled in the art. Such changes and modifications can be made without departing from the spirit and scope of the present invention and without diminishing its attendant advantages.

Although, for simplicity, this disclosure often makes references to rice it will be understood by those skilled in the art that the methods of the invention are also useful for the analysis of any plant species.

Mitogen-activated protein kinase (MAPK) plays a crucial role in regulating plant growth and development, as well as abiotic and biotic stress responses. However, until this invention very little was known about MAPK in monocot plants.

The present inventors isolated two alternatively spliced cDNAs, *OsMAPK5a* and *OsMAPKb* of a MAP kinase gene from rice. Alternative splicing of hnRNA is an important mechanism of gene expression and regulation.

The *OsMAPK5a* cDNA is 1396 base pairs (bp) long and encodes a predicted protein of 369 amino acids with an estimated molecular mass of 42.9 kilodaltons (kDa). The *OsMAPK5a* protein contains 11 subdomains that are conserved among all MAP kinase families (Hirt 1997) and possesses a dual phosphorylation activation motif TEY located between subdomains VII and VIII .

Therefore, the present invention provides an isolated nucleic acid molecule comprising the nucleotide sequence of *OsMAPK5a* (SEQ. ID.NO:1). This invention also includes an isolated nucleic acid molecule comprising a nucleotide sequence that encodes a polypeptide consisting of the amino acid sequence of SEQ. ID. NO: 2; or the complement of the nucleotide sequence of a polypeptide consisting of the amino acid sequence of SEQ. ID. NO: 2). The isolated nucleic acid molecule of *OsMAPK5a* (SEQ ID. NO: 2) includes cDNA or RNA. The present invention further includes an isolated nucleic acid molecule comprising a nucleotide sequence that encodes a polypeptide consisting of substantially the amino acid sequence of SEQ. ID. NO: 2; or the complement of the nucleotide sequence of a polypeptide consisting of substantially the amino acid sequence of SEQ. ID. NO: 2. The phrase "substantially the amino acid

sequence" refers to the MAPK5 ortholog of MAPK5 wherein the MAPK5 ortholog is MAPK5 having an identical amino acid sequence, or a polypeptide or polypeptide segment having a similar, non-identical sequence that is considered by those skilled in the art to be a functionally equivalent amino acid sequence. For example, an ortholog MAPK5 gene product having substantially the amino acid sequence of rice MAPK5 can include other monocot MAPK5 genes that are functionally equivalent to rice MAPK5 (SEQ ID NO:2). It is understood that minor modifications of primary amino acid sequence can result in an OsMAPK5-like gene product that has substantially equivalent or enhanced function as compared to the MAPK5 ortholog from which it was derived. Further, various molecules can be attached to an MAPK5 ortholog or active segment thereof, for example, other polypeptides, antigenic or other peptide tags, carbohydrates, lipids, or chemical moieties. Such modifications are included within the term MAPK5 ortholog as defined herein

The *OsMAPK5b* cDNA has an identical nucleotide sequence as that of the *OsMAPK5a* cDNA but lacks the 312 bp region from position 285 to 596 and encodes an incomplete MAP kinase with the deletion of subdomain III to VI .

The present invention provides also an isolated nucleic acid molecule comprising the nucleotide sequence of MAPK5b (SEQ. ID. NO: 3). The present invention also includes an isolated nucleic acid molecule comprising a nucleotide sequence that encodes a polypeptide consisting of the amino acid sequence of SEQ. ID. NO: 4; or the complement of the nucleotide sequence of a polypeptide consisting of the amino acid sequence of SEQ. ID. NO: 4. The isolated nucleic acid molecule of *OsMAPK5b* (SEQ. ID. NO: 4) includes cDNA or RNA. The isolated nucleic acid molecule of *OsMAPK5b* (SEQ ID. NO: 3) includes cDNA or RNA. The present invention further includes an isolated nucleic acid molecule comprising a nucleotide sequence that encodes a polypeptide consisting of substantially the amino acid sequence of SEQ. ID. NO: 4; or the complement of the nucleotide sequence of a polypeptide consisting of the amino acid sequence of SEQ. ID. NO: 4.

Altered nucleotide acid sequences may also be used in accordance with the present invention. Such altered nucleotide acid sequences include deletions, additions or substitutions of different nucleotide residues resulting in a sequence that encodes the

same or finally equivalent gene product of the present invention. The gene product itself may contain deletions, additions or substitutions of amino acid residues within the MAPK5 sequences. The DNA sequences of the invention may be engineered to alter the MAPK5 sequences for a variety of ends including, but not limited to alterations which modify processing and expression of the gene product. Mutations may be introduced using techniques well known in the art such as site-directed mutagenesis.

The present inventors generated transgenic rice plants with overexpression using the 35S promoter of Cauliflower mosaic virus and suppression using double-stranded RNA interference (dsRNAi) of OsMAPK5. However, a variety of expression systems may be utilized to express OsMAPK5 or ortholog MAPK5 nucleotide sequences of the present invention. Methods which are well known to those skilled in the art can be used to construct expression vectors containing the OsMAPK5 or ortholog MAPK5 coding sequence and appropriate transcriptional and/or translations control signals. These methods include but are not limited to *in vitro* recombinant DNA techniques, synthetic techniques and *in vivo* genetic recombination. Moreover, host cells containing OsMAPK5 or ortholog MAPK5 coding sequence may be identified by nucleic acid hybridization; the presence of or absence of marker genes, or immunoassays for detecting gene products or biological activity.

Therefore, one embodiment of the present invention provides an isolated nucleotide sequence consisting of MAPK5 linked to a heterologous protein or peptide. The present invention further provides recombinant vectors comprising the nucleotide sequence of MAPK5. In one embodiment, the recombinant vectors comprise the nucleotide sequences of *OsMAPK5a* (SEQ. ID. NO:1). In another embodiment, the recombinant vectors comprise the nucleotide sequences of *OsMAPK5b* (SEQ.ID.NO:3).

In yet another embodiment, the present invention provides a recombinant vector comprising an isolated nucleic acid molecule comprising a nucleotide sequence that encodes a polypeptide consisting of substantially the amino acid sequence of SEQ. ID. NO: 2; or the complement of the nucleotide sequence of a polypeptide consisting of the amino acid sequence of SEQ. ID. NO: 2. Another embodiment of the present invention provides a recombinant vector comprising an isolated nucleic acid molecule comprising a nucleotide sequence that encodes a polypeptide consisting of substantially the amino acid

sequence of SEQ. ID. NO: 4; or the complement of the nucleotide sequence of a polypeptide consisting of the amino acid sequence of SEQ. ID. NO: 4. It is understood that the complement of *OsMAPK5a* or *OsMAPK5b* or ortholog MAPK can be employed in this invention.

This invention includes an expression vector comprising the nucleotide sequence of SEQ.ID.NOs:1 or 2 or ortholog MAPK5 operatively associated with a regulatory nucleotide sequence containing transcriptional and translational regulatory information that controls expression of the nucleotide sequence in a host cell. The host expression vector system may include but not limited to microorganisms, insect, yeast or plants transformed with recombinant expression vectors.

The present invention provides a genetically host cell comprising the nucleotide sequences of SEQ. ID. NOs:1 or 3 or ortholog MAPK5. The genetically engineered host cell comprises an isolated nucleic acid comprising a nucleotide sequence that encodes a polypeptide consisting of the amino acid sequence of SEQ. ID. NO:2 or the complement of the nucleotide sequence of that encodes a polypeptide consisting of the amino acid sequence of SEQ. ID. NO:2. The genetically engineered host cell comprises an isolated nucleic acid comprising a nucleotide sequence that encodes a polypeptide consisting of the amino acid sequence of SEQ. ID. NO:4 or the complement of the nucleotide sequence of that encodes a polypeptide consisting of the amino acid sequence of SEQ. ID. NO:4. Moreover, the genetically engineered host cell of the present invention includes prokaryotic and eukaryotic cells.

In another embodiment, the genetically engineered host cell comprises the nucleotide sequences of SEQ. ID. NOs:1 or 3 or ortholog MAPK5 operatively associated with a regulatory sequence containing transitional and translational regulatory information that controls expression of the nucleotide sequences in the host cell.

The present invention further provides an isolated nucleic acid comprising a nucleotide sequence that encodes a polypeptide consisting of the amino acid sequence of SEQ. ID. NO:2 or the complement of the nucleotide sequence of that encodes a polypeptide consisting of the amino acid sequence of SEQ. ID. NO:2 operatively associated with a regulatory sequence containing transitional and translational regulatory information that controls expression of the nucleotide sequences in the host cell. The

present invention further provides an isolated nucleic acid comprising a nucleotide sequence that encodes a polypeptide consisting of the amino acid sequence of SEQ. ID. NO:4 or the complement of the nucleotide sequence of that encodes a polypeptide consisting of the amino acid sequence of SEQ. ID. NO:4 operatively associated with a regulatory sequence containing transitional and translational regulatory information that controls expression of the nucleotide sequences in the host cell. The genetically engineered host cell can be prokaryotic or eukaryotic. The host cell can be a continuous cell line.

This invention also demonstrated that the intact OsMAPK5a isoform has kinase activity. However, neither autophosphorylation nor MBP-kinase activity was detected for the truncated OsMAPK5b isoform. This is not surprising since OsMAPK5b is missing the subdomain VI which contains the catalytic loop of MAP kinase.

Therefore, the present invention provides a MAPK5 polypeptide having kinase activity. In one embodiment, this invention provides a polypeptide which has kinase activity comprising the amino acid sequence of OsMAPK5a (SEQ. ID. NO: 2) or ortholog MAPK. In still another embodiment, the ortholog MAPK polypeptide is selected from the group consisting of monocots or dicots.

The present invention relates to antibodies that are capable of specifically recognizing one or more OsMAPK5 or ortholog MAPK5 gene product epitope. Such antibodies may include but are not limited to polyclonal antibodies, monoclonal antibodies, humanized or chimeric antibodies, single chain antibodies, Fab fragments and epitope-binding fragments of any of the above. Immunoblot analysis using an antibody made against a 140 amino acid fragment of rice MAPK5 reacted with OsMAPK5a and OsMAPK5b.

In a preferred embodiment, the present invention provides an antibody that specifically binds to a peptide consisting of the C-terminal portion of the rice MAPK5 amino acid sequence set forth in SEQ. ID. NO:2. In another embodiment, the invention provides an antibody that specifically binds to a peptide consisting of the C-terminal portion of the rice MAPK5 amino acid sequence consisting of position 763 to the stop codon.

Further, the present invention provides an antibody that specifically binds to a peptide consisting of the C-terminal portion of the rice MAPK5 amino acid sequence set forth in SEQ. ID. NO: 4.

The present inventors detected MAPK protein by utilizing the immunoblotting technique. However, the detecting step of the present invention may be carried out by any suitable immunoassay, including homogeneous assays or heterogeneous assays. Examples of suitable immunoassays include but are not limited to radioimmunoassay, immunofluorescene assay, enzyme-linked immunosorbent assay (ELISA) and immunocytochemical assay.

The present invention provides a method for producing a transgenic plant with a MAPK5 or otholog coding nucleic acid, wherein expression of the nucleic acid in the plant results in increased tolerance to abiotic stress or resistance to biotic stress compared to a wild type plant comprising transforming a plant cell with an expression vector comprising the MAPK5 coding nucleic acid and generating a transgenic plant with an increased tolerance to abiotic stress or increased resistance to biotic stress compared to a wild type plant. In a preferred embodiment of the present invention, *Agrobacterium* can be employed to introduce the gene constructs into plants.

The present invention provides a transgenic plant transformed with a nucleotide sequence that encodes a MAPK5 or MAPK5 ortholog nucleic acid sequence wherein overexpression of the MAPK5 ortholog nucleic acid sequence in the plant results in increased tolerance to abiotic stress compared to a wild-type plant. In another embodiment, the transgenic plant is transformed by a nucleotide sequence that encodes a polypeptide consisting of the amino acid sequence of SEQ. ID. NO: 2 wherein overexpression of SEQ. ID. NO: 2 in the plant results in increased tolerance to abiotic stress compared to a wild-type plant. Abiotic stress includes but not limited to drought, temperature and salinity.

In yet another embodiment, the transgenic plant is transformed by a nucleotide sequence that encodes RNA interference structure wherein suppression of the MAPK5 ortholog nucleic acid sequence in the plant results in increased resistance to biotic stress compared to a wild-type plant. In still another embodiment, the transgenic plant is transformed by a nucleotide sequence that encodes a polypeptide consisting of the amino

acid sequence of SEQ. ID. NO:2 wherein suppression of the expression of SEQ. ID. NO:2 in the plant results in increased resistance to biotic stress as compared to wild-type plant. Biotic stress includes but not limited to infection or disease generated by pathogenic fungi, bacteria, viruses, nematodes and insects.

The present invention further provides a transgenic plant transformed by a nucleotide sequence that encodes a MAPK5 ortholog nucleic acid sequence operatively linked to a regulatory sequence that controls gene expression so that the MAPK5 ortholog nucleic acid sequence is overexpressed in the plant compared to a wild-type plant. In another embodiment, the transgenic plant is transformed by a nucleotide sequence that encodes a polypeptide consisting of the amino acid sequence of SEQ. ID. NO: 2 operatively linked to a regulatory sequence that controls gene expression so that SEQ. ID. NO: 2 is overexpressed in the plant compared to a wild-type plant. The transgenic plant is further transformed by a nucleotide sequence that encodes a MAPK5 ortholog nucleic acid sequence operatively linked to a regulatory sequence that controls gene expression so that expression of the MAPK5 ortholog nucleic acid sequence is suppressed in the plant compared to a wild-type plant.

In another embodiment, the transgenic plant is transformed by a nucleotide sequence that encodes a polypeptide consisting of the amino acid sequence of SEQ. ID. NO: 2 operatively linked to a regulatory sequence that controls gene expression so that expression of SEQ. ID. NO: 2 is suppressed in the plant compared to a wild-type plant.

The transgenic plant of this invention comprises MAPK5 nucleic acid from rice. In another embodiment, the transgenic plant of this invention comprises MAPK5 nucleic acid from a monocot other than rice. Monocots include but not limited to wheat, barley, rice and maize.

The present also includes for seeds produced by the transgenic plants of this invention.

This invention demonstrated for the first time that an ABA-inducible rice MAP kinase is capable of inversely modulating disease resistance and abiotic stress tolerance. On one hand, overexpression of *OsMAPK5* resulted in enhanced plant tolerance to drought, salt and cold stresses while on the other hand, suppression of OsMAPK5 reduced abiotic stress tolerance, but led to constitutive *PR* gene expression and increased

disease resistance. Therefore, this invention further provides methods for evaluating tolerance to abiotic stress or resistance to biotic stress. For example, one method provides for evaluating a plant for tolerance to abiotic stress comprising treating a plant with abiotic stress; isolating MAPK5 protein from the plant; detecting for MAPK5 activity; and evaluating the increase or decrease in MAPK5 activity in the plant whereby the increase in MAPK5 activity indicates the plant is tolerant to abiotic stress. Abiotic stress includes infection or disease from pathogenic fungi, bacteria, viruses, nematodes and insects. MAPK5 or its ortholog is isolated by immunoprecipitating the protein with a MAPK5 protein that specifically binds to MAPK5.

In another embodiment, the method of this invention provides for evaluating a plant for resistance to biotic stress comprising treating a plant with a pathogen; isolating MAPK5 protein from the plant; detecting for MAPK5 activity; and evaluating the increase or decrease in MAPK5 activity in the plant whereby the decrease in MAPK5 activity indicates the plant is tolerant to the pathogen. Biotic stress includes drought, temperature and salinity.

In yet another embodiment, the method of this invention provides for enhancing tolerance to abiotic stress in a plant comprising transforming a plant with MAPK5 nucleic acid sequence wherein the MAPK5 protein is expressed in the plant; treating a plant with an abiotic stress; isolating MAPK5 protein from the plant; detecting for MAPK5 activity; and evaluating the increase or decrease in MAPK5 activity in the transformed plant whereby the increase in MAPK5 activity indicates the increase in tolerance to abiotic stress in the transformed plant compared to the wild-type plant.

The studies using the present invention demonstrated that rice *PR* genes such as *PR-1b* and *PR-10*, which are involved in disease resistance, were constitutively activated in both young seedlings and mature *OsMAPK5-RI* transgenic lines under normal growth condition. In still another embodiment, the method of this invention provides for increasing resistance to biotic stress in a plant comprising transforming a plant with MAPK5 nucleic acid sequence wherein the MAPK5 protein is expressed in the plant; treating a plant with a biotic stress; isolating MAPK5 protein from the plant; detecting for MAPK5 activity; and evaluating the increase or decrease in MAPK5 activity in the

transformed plant whereby the decrease in MAPK5 activity indicates the increase resistance biotic stress in the transformed plant compared to the wild-type plant.

The present also provides kits for screening plants for susceptibility to biotic stress or tolerance to abiotic stress. One kit includes an isolated nucleic acid probe that comprises a label and (a) nucleotide sequence that encodes a polypeptide consisting essentially of the amino sequence of SEQ. ID. NO:2 or (b) the complement of (a). In another embodiment, the kit includes an isolated nucleic acid probe that comprises a label and (a) nucleotide sequence that encodes a polypeptide consisting of essentially the amino sequence of SEQ. ID. NO:4 or (b) the complement of (a).

The kit of the present invention provides for screening a plant for susceptibility to biotic stress comprising a nucleic acid probe and at least one reagent suitable for detecting the presence of a nucleic acid molecule encoding MAPK5 whereby the changes in polymorphic patterns of MAPK5 indicates the plant is susceptible to biotic stress. .

Another kit of the present invention provides for detecting a plant for tolerance to abiotic stress comprising an antibody that immunospecifically binds to a MAPK5 polypeptide wherein the antibody is labeled; and at least one reagent suitable for detecting the presence of MAPK5 whereby the increase or decrease in MAPK5 activity indicates the plant is tolerant to abiotic stress.

The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention.

7. MATERIALS AND METHODS

7.1 Isolation and Sequence Analysis of OsMAPK5

A full-length *OsMAPK5* cDNA was isolated using a **231?** base pair *OsMAPK5* cDNA fragment (JB113) as a probe (Xiong et al., 2001). Approximately 106 plagues from a blast–induced cDNA library (Lee et al. 2001) were screened. The resulting positive clones carrying *OsMAPK5* cDNAs were excised *in vivo* from the lambda ZAP express vector with the aid of ExAssist helper phage (Startagene, La Jolla, CA). The full-length *OsMAPK5* cDNA clones were sequenced from both directions by a primer walking approach. Automated sequencing service was provided by the University of Arkansas for Medical Science. Sequence analysis was

transformed plant whereby the decrease in MAPK5 activity indicates the increase resistance biotic stress in the transformed plant compared to the wild-type plant.

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7. MATERIALS AND METHODS

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performed using Vector NT1 Suite (Informax, North Bethesda, MD) and BLAST (Altschul et al., 1990).

7.2 Gene Construction

7.2.1 Construction of OsMAPK5-OX

An overexpression construct, *OsMAPK5-OX* was constructed by digesting the full length cDNA *OsMAPK5* with *Bam*HI and *Xba*I and directionally inserting into pCAMBIA1300S, a modified pCAMBIA1300 vector that contains a double CaMV 35S promoter and a terminator.

7.2.2 Construction of *OsMAPK5-RI*

A doubles-stranded RNA interference (dsRNAi) construct was made by generating antisense and sense fragments of the *OsMAPK5* cDNA using restriction enzyme digestions and PCR methods. The antisense fragment spanning nucleotides from 1198 to 1 of *OsMAPK5* including 6 bases from the vector of pBK-CMV was obtained by digestion with *Nco*I and *Bam*HI and inserted into a *Nco*I and *Bam*HI site of pCMBIA1300S to form an antisense construct, pC1300S-A. The sense fragment spanning nucleotides from 734 to 1198 of *OsMAPK5* was generated by PCR with primers B734 containing a *Bam*HI site (5'-CGGGATCCGTCGGCTGCATCTTCATG) (SEQ. ID. NO:5) and X1198 containing a *Xba*I site (5'-GCTCTAGATTCAATCTAGTACCGGA) (SEQ. ID. NO:6). The PCR product was digested by *Bam*HI and *Xba*I and inserted into the *Bam*HI and *Xba*I site of pC1300S-A to form the dsRNAi construct, *OsMAPK5-RI*.

7.3 Transformation

Overexpression and dsRNAi constructs, *OsMAPK5-OX* and *OsMAPK5-RI* were separately introduced into *Agrobacterium tumefaciens* (strain EHA105) by a freeze-thaw method (Hofgen and Willmitzer, 1998). pCAMBIA1300S vector only transformed plants were used as controls. *Agrobacterium tumefaciens* carrying overexpression or dsRNai constructs was grown overnight in AB induction medium (Winans et al., 1988) containing 50 μg/ml hygromycin and 100 μM acetosyringone. Bacterial cells were collected by centrifugation and resuspended in AB induction medium to an OD_{600} of 0.1. The *Agrobacterium tumefaciens* rice transformation was performed according to Hiei et al. (1994) by vigorously growing calli derived from

mature embryos of Nipponbare GA3 (*Oryza sativa* L.), a cultivar that is used in the international rice genome sequencing project and relatively easy for transformation.

Although, the present invention employs *Agrobacterium* to introduce gene constructs into plant tissue and cells, one skilled in the art may employ other techniques. Alternatively, recombinant nucleic acid sequences may also be introduced into plants and plant cells by gene transfer and transformation methods including but not limited to, protoplast transformation, electroporation-mediated uptake of naked DNA and electroporation of plant tissues or plant cell transformation through microinjection.

7.4 Plant Material

Transgenic rice plantlets at 5-6 cm in height were transplanted into Scott® Redi-earth and grown at 28°C in the greenhouse with a 14/10 hour light/dark cycle. The plants were fertilized with 0.5% ammonium sulfate every two weeks until flowering. Self-pollinated seeds from independent transgenic lines were harvested. T_1 plants carrying the transgene were selected by germinating seeds on filter paper soaked with 50 μg/ml hygromycin. Non-transgenic seeds of Nipponbare GA3 (*Oryza sativa* L.) did not germinate in the presence of 50 μg/ml hygromycin. Positive T_1 plants were confirmed by PCR or Southern analysis using primers or a probe corresponding to the 35S promoter and/or the 5′ region of *OsMAPK5*. Wild–type and transgenic plants of Nipponbare GA3 (*Oryza sativa* L.) cultivar and the U.S. rice cultivar Drew were used for *M. grisea* infection.

7.5 Pathogen Inoculations

The fungal isolates of the IC-17 pathotype of *P. grisea* were used. On cultivar Drew carrying the *Pita* resistance gene, the IC17-18/1 isolate carrying *avrPita* is avirulent. However, its race-change mutant IC17-18/1-2, lacking *avrPita* is virulent (Harp and Correll, 1998). Both isolates are virulent on cultivar Nipponbare. The fungal infection of T_0 transgenic plants was carried out using the spot inoculation method (Jin and Valent, 2001). Leaf segments (5-6 cm long) were isolated from the top of the full-expanded leaf and placed in a Petri dish on a circular filter paper soaked with water. Droplets containing about 50 spores in 0.02% Tween-20 were applied to the leaf surface. The Petri dishes were covered and maintained at

24°C under white light. Visual evaluation of disease systems and quantification of fungal growth were conducted at 5 or 6 days post-inoculation. The fungal infection of two-week-old T_1 and T_2 transgenic plants was carried out using the typical spray-inoculation method at a concentration of 250,000 spores per ml (Lee et al., 2001). Blast resistance was evaluated based on the fungal growth *in planta* (Qi and Yang, 2002) as well as lesion number and size.

Control and transgenic plants were inoculated with a virulent strain of *Burkholderia glumae*, the casual agent of bacterial sheath rot or panicle blight diseases by injecting 20 µl of bacterial suspension (ca. 10^6 cfu/ml) into sheaths of one-moth-old rice plants. Host resistance to bacterial infection was evaluated based on the severity of disease symptoms as well as the levels of bacterial growth *in planta*.

7.6 Chemical and Abiotic treatments

Chemical treatments were conducted on two-week-old seedlings by spraying with abscisic acid (ABA) (0.1 mM), jasmonic acid (JA) (0.1mM) or salicyclic acid (SA) (1 mM) solutions. Mechanical wounding was achieved by crushing rice leaves with a hemostat.

Abiotic treatments and evaluations were conducted according to Saijo et al (2000). Seedlings were grown in large flat trays rather than individual pots to minimize potential variations among different pots.

Cold stress treatment was performed by transferring seedlings to 4°C for 3 days and returning to normal growth conditions for recovery.

Drought stress was obtained by withholding water for up to 6 days. Using greenhouse conditions of 28°C on a 14 h/8 h light/dark cycle and two week old seedlings, leaves began to wilt three days after the free water was removed.

Salt stress treatment was performed by immerging roots of two week old seedlings in 200 mM NaCl solution for up to 4 days. The stressed plants were returned back to normal growth conditions when approximately half of the control plant became wilted. The levels of cold, drought or salt tolerance were evaluated based on the percentage of survived seedlings after a period of recovery.

7.7 Southern and Northern Blot Analysis

Four micrograms of genomic DNA isolated by the CTAB method (Zhang et al., 1992) from the cultivar Drew were digested individually with *Eco*RI, *Hind*III, *Pst*I and *Xba*I; fractionated on a 0.7% agarose gel and blotted onto a nylon membrane (Sambrook et al., 1989). Total RNA was isolated from rice leaves using TRIzol reagent (Life Technologies, Rockville, MD). Fifteen micrograms of total RNA from each sample were separated on a 1.2% agarose gel containing formaldehyde and then transferred onto a nylon membrane. DNA and RNA ladders (Promega) were added in the gels to estimate the sizes of hybridized bands. DNA or RNA blots were hybridized with a [α-^{32}P] dCTP-labeled gene-specific probe of the sequence from the 999th nucleotide to the 3'-end of *OsMAPK5*a cDNA in PerfectHyb buffer (Sigma). Hybridization and washing conditions were based on the manufacturer's instructions.

Two gene-specific primers, 5'-GAGTTCAGGCCGACGATGAC-3' (RT-F99) (SEQ. ID. NO:7) and 5'-ATCGGCGATGTCGT GCAATC-3' (RT-R1067) (SEQ. ID. NO:8), were designed for amplifying DNA fragments covering the differentiated region of *OsMAPK5a* and *OsMAPK5b* transcripts. Rice genomic DNA and reversely transcribed cDNAs from the blast fungus-induced total RNA (two days after infection) were used as templates for the Polymerase Chain Reaction (PCR) analysis.

7.8 Recombinant Protein Production

A *Bam*HI site was introduced to *OsMAPK5* at the start codon using Quickchange site-directed mutagenesis (Stratagene). The entire coding region of *OsMAPK5* was digested with *Bam*HI and *Xho*I and ligated in-frame into the His-tag of pET-28(+) vector (Novagen). A specific *OsMAPK5* antigen was generated by digesting a DNA fragment spanning from nucleotide positions 763 to the 3'-end of *OsMAPK5* with *Sac*I and *Xho*I and ligating in-frame into the His-tag of pET-28a(+).

7.9 Antibody Production

Recombinant proteins were induced and purified from *E. coli* cells according to the manufacturer's instruction (Pierce). Polyclonal antisera against a 140 amino acid C-terminal region of *OsMAPK5* were raised in rats. Antibodies also may be generated from other animals such as but not limited to, rabbits, mice or chickens by known techniques. Antibodies may include, but are not limited to, polyclonal antibodies, monoclonal antibodies, humanized chimeric antibodies, single chain

antibodies, Fab fragments and epitope-binding fragments of any of the above.

7.10 Autophosphorylation Assay

The autophosphorylation assay was conducted according to Huang et al. (2000). Purified recombinant *OsMAPK5* protein (300 ng) in reaction buffer (40 mM Hepes pH 7.5, 20 mM $MgSO_4$, 10 mM $MnCl_2$, 1 mM $CaCl_2$, 200 mM ATP and 10 μCi γ-^{32}P-ATP) was incubated for 1 hour at room temperature. The reaction mixture was stopped by the addition of SDS sample buffer and heating at 80°C for 10 minutes. After separation on a 10% SDS-PAGE gel, the phosphorylated product was detected by autoradiography.

7.11 Protein Extraction and Immunoblotting

Rice leaf tissues were ground in liquid nitrogen and homogenized in extraction buffer containing 50 mM Tris (pH 8.0), 1 mM EDTA, 6 mM β–mercaptoethanol, 0.5 mM phenyl-methylsulfonyl fluoride (PMSF) and 0.3 M aprotinin. After centrifugation at 16, 000 g, aliquots of supernatant were frozen immediately in liquid nitrogen and stored at -80°C. The protein concentration was determined by using a Bio-Rad protein assay kit with bovine serum albumin (BSA) as a standard.

Equal amounts of protein extracts were separated on 12% SDS polyacrylamide gels and electro-transferred onto nitrocellulose membranes in a transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3). Non-specific binding sites were blocked by incubating the membrane in 1xTBS-T (25 mM Tris, 140 mM NaCl, 0.1% Tween-20, pH 7.5) containing 6% non-fat dry milk for 1 hour at room temperature. Anti-*OsMAPK5* antibody at 1:8000 dilution was added and the membranes were incubated overnight at 4°C. After rinsing 3 times for 15 minutes each with 1xTBS-T, the membrane was incubated with HRP-conjugated anti-rat IgG antibody at 1:1000 dilution (Sigma) in TBS-T buffer for 1 hour at room temperature. Following 5 washes for 15 minutes each with TBS-T buffer, the *OsMAPK5* protein was detected with the ECL Plus diction system (Amersham). Biotinylated protein standards were separated in the same gel and detected by Avidin-HRP conjugate (Bio-Rad) as a size marker.

7.12 Immunoprecipitation and In-gel Kinase Activity Assay

Approximately 0.4 milligrams of protein extracts were incubated with 50 µl of anti-*OsMAPK5* antibody at 4°C overnight. Fifty microliters of protein G agarose bead was added and incubated for 2 hours at 4°C. The protein-antibody complex was collected and washed three times in ice-cold phosphate-buffered saline and resuspended in protein sample buffer.

The in-gel kinase activity assay was performed as described by Zhang and Klessig (1997) with some modifications. Forty micrograms of total protein or immunoprecipitate from 400g of total protein was fractionated on a 10% polyacrlamide gel containing 0.1% SDS and 0.25% mg/ml bovine brain myelin basic protein (MBP, Sigma). SDS was removed by washing the gel three times for 30 minutes each at room temperature with buffer containing 25 mM Tris, pH 7.5, 0.5 mM DTT, 0.1 mM Na_3VO_4, 5 mM NaF, 0.5 ma/ml BSA, 0.1% Triton X-100. The kinases were allowed to renature overnight at 4°C with three changes of renature buffer (25 mM Tris, pH 7.5, 1 mM DTT, 0.1 mM Na_3VO_4, 5 mM NaF). The phosphorylation of MBP was performed in a 30 milliliter reaction buffer (25 mM Tris, pH 7.5, 2 mM EGTA, 12 mM $MgCl_2$, 1 mM DTT, 0.1 mM Na_3VO_4) with the addition of 0.2 M ATP and 50 µCi γ-^{32}P-ATP (3000 Ci/mmol) at room temperature for 60 minutes. The gel was transferred to washing buffer (5% trichloroacetic acid, 1% sodium pyrophosphate) at room temperature for at least 5 hours with five buffer changes.

8. EXAMPLES

The invention having been described, the following examples are offered by way of illustration and not limitation.

8.1 Isolation and Sequence Analysis of *OsMAPK5* cDNAs

A rice cDNA fragment (JB113) was previously identified to be inducible by blast fungus, *M. grisea* (Xiong et al. 2001). Full-length cDNA clones were isolated from a rice cDNA library using the JB113 cDNA fragment as a probe. Two full-length *OsMAPK5* cDNAs that are alternatively spliced from a single gene were isolated and designated as *OsMAPK5a* and *OsMAPK5b*.

The *OsMAPK5a* cDNA (accession number AF479883) is 1396 base pairs (bp) long and encodes a predicted protein of 369 amino acids (Figure 1A) with an estimated molecular mass of 42.9 kilodaltons (kDa). The *OsMAPK5a* protein contains 11 subdomains that are conserved among all MAP kinase families (Hirt 1997) and possesses a dual phosphorylation activation motif TEY located between subdomains VII and VIII (Figure 1A). The protein shares the identical amino acid sequence encoded by *OsMSRM2* (Agrawal el al., 2002), *OsMAPK2* (Huang el., 2002), *OsMAP1* (Wen et al., 2002) and *OsMIMK1* (Song et al., 2002). The *OsMAPK5a* protein also shares a very high homology of 91% identity with the elicitor-inducible *TaWCK-1* (Takezawa et al., 1999) from wheat and 73% identity with the wound-inducible *NtWIPK* (Seo et al., 1995) from tobacco.

Phylogenetic analysis based on sequence alignment of the catalytic domain suggests that *OsMAPK5a* belongs to the A1 subgroup of plant MAP kinase family (Figure 1B). The phylogenetic relationship of *OsMAPK5a* and OsMAPK5b was compared to other plant MAPKs. The dendrogram was constructed using Vector NTI Suite software (Informax, North Bethesda, MD). For simplicity, representatives from the eight subgroups of plant MAPKs, including a few putative rice MAPKs, were included in the dendrogram. The accession numbers for the MAPKs shown in the figure are as follow: AtMPK3, D21839; NtWIPK, D61377; MsMMK4, T09622; TaWCK-1, AF079318; OsMAPK5a, AF479883; ZmMPK4, AB016801; AtMPK6, D21842; NtSIPK, U94192; AtMPK4, D21840; AtMPK5, D21841; AtMPK13, AAF75067; AtMPK1, D14713; AtMPK7, D21843; OsMAPK3, AF216317; OsMAPK4, AJ251330; AtMPK8, AB038693; OsBWMK1, AF177392; OsRMAPK2, AF194416. Previous studies indicate that members of the A1 and A2 subgroups are frequently activated by various biotic and abiotic stresses (Zhang and Klessig, 2001).

The *OsMAPK5b* cDNA (accession number AF479884) has an identical nucleotide sequence as that of the *OsMAPK5a* cDNA except that a 312 bp region from position 285 to 596 is deleted. The *OsMAPK5b* encodes an incomplete MAP kinase with the deletion of subdomain III to VI (Figure 1A).

8.2 Genetic Analysis of *OsMAPK5*

To determine whether *OsMAPK5a* and *OsMAPK5b* were derived from alternative splicing of a single gene, Southern hybridization was performed using a probe covering an identical region of *OsMAPK5a* and *OsMAPK5a* (nucleotide 999 to the 3'-end of *OsMAPK5a)*. One strongly hybridizing band was detected in rice genomic DNA digested with *Eco*RI, *Hind*III, *Pst*I, and *Xba*I, respectively (Figure 2A). Genomic PCR of rice genomic DNA using two primers that covered the differentiated region also gave rise to a single fragment (data not shown). However, RT-PCR with the same pair of primers amplified two cDNA fragments from the blast fungus-induced RNA sample. Molecular sizes of 1.0 and 0.6 kb matched consistent with the predicted sizes of the cDNA fragments based on the location of the two primers (Figure 2B). Therefore, *OsMAPK5a* and *OsMAPK5b* most likely resulted from the alternative splicing of a single *OsMAPK5* gene in rice. There is a low-level expression of *OsMAPK5* in normal, uninfected leaves, as detected by RT-PCR (data not shown). In both uninfected and infected leaf tissues, *OsMAPK5a* was a predominant isoform of *OsMAPK5* transcripts.

8.3 Analysis of Kinase Activity in OsMAPK5a and OsMAPK5b

To determine whether *OsMAPK5a* and *OsMAPK5b* encode active MAP kinases, the recombinant proteins of both OsMAPK5a and OsMAPK5b were produced and purified from *E. coli* cells harboring *OsMAPK5a* and *OsMAPK5b* coding sequences in the expression vector pET-28c(+), respectively. As expected, OsMAPK5b was 12 kDa smaller than OsMAPK5a as a result of 312 bp or a 104 amino acid deletion (Figure 2C). Kinase assays revealed that only OsMAPK5a exhibited autophosphorylation activity, suggesting that the missing subdomains in OsMAPK5b are essential for the kinase activity (Figure 2D).

8.4 Induction of *OsMAPK5* by *M. grisea* infection

Previous study by the present inventors revealed that *OsMAPK5* was inducible by the blast fungus (Xiong et al., 2001). To further assess the expression pattern of *OsMAPK5* during fungal infection, an avirulent blast isolate carrying *AvrPita* and its virulent mutant lacking *AvrPita* were used to elicit resistant and susceptible reactions, respectively, on rice cultivar Drew, *Oryza sativa spp. japonica,* carrying the *Pita* resistance gene. RNA blots prepared from mock-treated and blast-infected leaves were hybridized with a gene-specific probe of *OsMAPK5*. Two hybridizing transcripts were

determined to be induced by the blast fungus (Figure 3A). The sizes of the transcripts were similar to those of *OsMAPK5a* and *OsMAPK5b* cDNAs at 1.4 and 1.1 Kb, respectively. However, the induced level of *OsMAPK5b* transcripts was significantly lower than that of *OsMAPK5a*. In the resistant interaction, the mRNA level of *OsMAPK5* was induced as early as one day after inoculation, peaked on the second day and then declined (Figure 3A). In the susceptible interaction, the transcripts accumulated slowly, but lasted longer than in the resistant interaction. However, the peak level of induced *OsMAPK5* was significantly higher in the resistant interaction than in the susceptible interaction. No induction of *OsMAPK5* was detected in mock-treated leaves indicating that induction of *OsMAPK5* was not due to the effect of spray inoculation (Figure 3A).

Using anti-OsMAPK5 antibody, a 43 kDa protein corresponding to OsMAPK5a with a predicted size 42.9 kDa was detected in rice leaves infected with *M. grisea* (Figure 3B). Immunoblot analysis indicated that the level of OsMAPK5a protein increased slightly on the second day after the infection with avirulent isolate and then dropped to the base level. In the susceptible reaction, however, much more protein was induced and the induction lasted longer (Figure 3B). The OsMAPK5b protein with a predicted size 31.2 kDa was undetectable using the same experimental conditions for detecting OsMAPK5a (5 to 10 min of exposure time using the ECL-Plus detection kit). A rather weak band corresponding to OsMAPK5b was detected under extended exposure time of more than 1 hour. An unknown constitutively expressed protein with a molecular weight of 49 kDa cross-reacted with the anti-OsMAPK5 antibody (data not shown).

To further examine whether the OsMAPK5a kinase activity was induced by blast infection, the endogenous OsMAPK5a was immunoprecipitated and subjected to in-gel kinase assay using myelin basic protein (MBP) as a substrate. Results showed that the OsMAPK5a kinase activity was significantly induced by *P. grisea* infection. In the resistant interaction, the kinase activity increased one day after fungal inoculation and then declined progressively to the base level. In the susceptible interaction, the kinase activity increased after 2 days, but remained moderately high until the final stage of infection (Figure 3C). Since neither MBP kinase activity (Fig 3C) nor autophosphorylation activity (Fig 2D) was detected for OsMAPK5b, only the band

corresponding OsMAPK5a was shown in the immunoblot analyses. These data suggest that the early transient activation of OsMAPK5a activity is probably related to the resistance response to avirulent blast isolates. The constant activation of OsMAPK5a in the later stage of infection, on the other hand, may be related to stress resulting from the development of the disease.

8.5 Induction of *OsMAPK5* by ABA and wounding

To determine the effects of different signaling molecules on *OsMAPK5* activation, two-week-old rice seedlings were treated with abscisic acid (ABA), salicylic acid (SA) and jasmonic acid (JA). RNA blot analysis revealed that the *OsMAPK5a* was significantly induced in rice leaves treated with 0.1 mM ABA (Figure 4A). Transcripts of *OsMAPK5a* quickly accumulated to the highest level at 2 hours after treatment and then declined. However, *OsMAPK5a* was only slightly induced, if at all, in leaves treated with 1 mM SA or 0.1 mM JA. Moreover, treatments with higher concentration of SA or JA did not significantly induce *OsMAPK5a* (data not shown). In contrast, a defense-related gene *PR-10* was induced by SA and JA as expected (Figure 4A). Expression of *OsMAPK5a* increased significantly in wounded leaves, peaking at 30 min after wounding and then decreasing rapidly to the base level (Figure 4A). The transcript of *OsMAPK5b* was not induced by all these chemical treatments or wounding.

Immunoblot analysis revealed that the OsMAPK5 protein was induced by ABA and wounding but not by SA or JA (Figure 4B). The immunocomplex in-gel kinase assay also revealed that OsMAPK5 activity was induced by ABA and wounding but not by SA or JA (Figure 4C). After ABA treatment, the peak of OsMAPK5 activity appeared earlier than that of the mRNA and protein. Similar phenomena were also observed following the fungal infection (Figure 3) or abiotic treatments (Fiure 5). Previously, Seo et al. (1995) reported that the peak of tobacco WIPK activity appeared much earlier than that of its mRNA after mechanical wounding. It is very likely that the basal level OsMAPK5 can be activated very quickly before the accumulation of its mRNA and protein.

8.6 Induction of *OsMAPK5* by drought, salinity and low temperature

RNA blot analysis revealed that *OsMAPK5a* was induced by drought, salinity or low temperature (Figure 5A). In the drought and salt treatments, *OsMAPK5a* was

induced earlier in roots or within 1 day and an 1 hour for drought and salinity, respectively than in leaves within 4 days and 3 hours for drought and salinity, respectively. The transcript of *OsMAPK5a* remained high throughout the course of drought stress. Under salt stress, however, the transcripts declined at 6 hours after the treatment. The transcript of *OsMAPK5a* was also inducible within 6 hours by low temperature (4°C) treatment (Figure 5A).

Immunoblot analyses revealed that the protein level of OsMAPK5 was significantly increased in rice seedlings under drought and salt stresses, but was slightly induced by low temperature (Figure 5B). Immunocomplex kinase assay indicated that OsMAPK5 activity was also induced by drought, salt and low temperature (Figure 5C). These results suggest that OsMAPK5 is likely involved in abiotic stress responses in rice plants.

8.7 Overexpression of OsMAPK5 in transgenic rice

To clarify the role of *OsMAPK5a* in biotic and abiotic stress responses, the expression of *OsMAPK5* was constitutively increased or suppressed in transgenic rice. The transgenic lines were generated by introducing the overexpression construct, *OsMAPK5-OX* or the double-stranded RNA interference construct, *OsMAPK5-RI* into cultivar Nipponbare GA3.

A total of 30 independent overexpression lines were generated using the *OsMAPK5-OX* construct. Southern analysis indicated that 19 *OsMAPK5-OX* lines contained a single-copy insertion (data not shown). RNA blot analysis showed that the *OsMAPK5* gene was expressed constitutively in transgenic lines but not in the control plants under normal growth conditions (5 lines are shown in Figure 6A as examples). As expected, the protein of OsMAPK5a was constitutively produced in the transgenic lines but not in the control plants under normal growth conditions (Figure 6A). However, the MBP kinase activity of OsMAPK5a in these lines was not significantly increased (Figure 6A). All the *OsMAPK5-OX* lines showed no obvious phenotypic changes in comparison with control plants throughout the life cycle.

8.8 Suppression of *OsMAPK5* in transgenic rice

A total of 38 independent suppression lines were generated using the *OsMAPK5-RI* construct. Twenty-four *OsMAPK5-RI* lines were confirmed by Southern hybridization

to carry a single-copy insertion (data not shown). RNA blot analysis showed that *OsMAPK5-RI* construct was constitutively transcribed in suppression lines (5 lines are shown in Figure 6B as examples). Since the endogenous level of OsMAPK5 in control plants is rather low under normal growth conditions (Figure 3C), the effectiveness of dsRNAi in T_0 transgenic lines was examined after induction of OsMAPK5 by spot inoculation of rice leaves with the blast fungus. Strikingly, the production of endogenous OsMAPK5 protein was almost completely blocked even under the induced condition. In fact, no MBP kinase activity was detected for OsMAPK5 in these transgenic lines (Figure 6B). The suppression of endogenous OsMAPK5 by dsRNAi was also transmitted to T_1 transgenic plants (see Section 7.7). None of the *OsMAPK5-RI* lines showed obvious phenotypic changes from germination to the early vegetative growth stage. However, starting from the late vegetative stage of about 2 months after germination, irregular brownish stripes developed on the mature leaves of *OsMAPK5-RI* lines (Figure 6C). Nevertheless, each *OsMAPK5-RI* lines proceeded to the reproductive stage and had normal seed setting.

When conducting the in-gel kinase assay using leaf protein extracts from the blast fungus-infected seedlings, kinase activity of a 37 kDa protein was significantly increased in *OsMAPK5-RI* transgenic plants but not in the control plants (unpublished data). This data suggests a potential antagonistic effect of OsMAPK5 on an unknown MBP kinase that may positively regulate defense response in rice.

8.9 Negative regulation of broad-spectrum host resistance by *OsMAPK5*

The effects of overexpression or suppression of *OsMAPK5* on host resistance to fungal and bacterial pathogens was examined. Disease resistance was initially evaluated on first generation (T_0) transgenic lines by spot inoculation of transgenic leaves with a virulent isolate of *M. grisea* because single T_0 plants were not suitable for spray inoculation. Both control and *OsMAPK5-OX* T_0 lines exhibited the same level of disease susceptibility to blast infection with the average lesion sizes of 7.0 ±1.2 mm and 6.8 ±1.6 mm, respectively. But all *OsMAPK5-RI* T_0 lines (20 lines tested) exhibited significantly enhanced resistance with average lesion size of 2.8 ± 1.1 mm. Fungal growth that was quantified based on relative rRNA contents of *M. grisea* in inoculated spots was also

reduced about three to six fold in *OsMAPK5-RI* lines compared to control or *OsMAPK5-OX* lines.

To confirm the results from the T_0 generation, the disease resistance in the second generation (T_1) of transgenic rice using three *OsMAPK5-OX* lines, four *OsMAPK5-RI* lines and the control line was evaluated. Due to the transgene segregation in the T_1 generation, seedlings carrying the *OsMAPK5-OX* or *OsMAPK5-RI* constructs were first identified based on hygromycin resistance and positive PCR amplification of the transgene. Approximately 40 two-week-old T_1 seedlings from each line (a total of more than 320 seedlings) were spay-inoculated with the fungal isolate (IC17-18/1). As indicated by significantly reduced disease severity (Figure 7A and 7B), lesion numbers (Figure 7C) and fungal growth (Figure 7D), all four *OsMAPK5-RI* lines demonstrated increased resistance to blast infection. In contrast, the control and *OsMAPK5-OX* plants were very susceptible to the same fungal isolate. As expected, the normal induction of OsMAPK5 kinase activity by fungal infection was almost completely suppressed in these *OsMAPK5-RNAi* lines (Figure 7E), suggesting that suppression of OsMAPK5 activity likely led to the enhanced resistance.

To test whether *OsMAPK5-RI* lines have broad-spectrum resistance to other pathogens, four-week-old T_1 plants were infected with *Burkholderia glumae*, a bacterial pathogen causing rice diseases known as panicle blight, glume blight or sheath rot complex (Cottyn et al., 1996). In comparison with the control or *OsMAPK5-OX* lines, *OsMAPK5-RI* lines exhibited significantly elevated resistance against the bacterial pathogen as indicated by reduced lesion size (Figure 8A) and bacterial growth (Figure 8B). The OsMAPK5 kinase activity was activated by *B. glumae* in both control and *OsMAPK5-OX* plants but was again suppressed in *OsMAPK5-RI* lines (Figure 8C). These results demonstrate that suppression of OsMAPK5 activity in rice may result in broad-spectrum resistance to fungal and bacterial pathogens.

In all the tests, the control and *OsMAPK5-OX* plants demonstrated no significant difference in host susceptibility to either *M. gisea* or *B. glumae* (Figures 7B and 8B). Although the OsMAPK5 protein was constitutively expressed in the *OsMAPK5-OX* lines (Figure 6B), the kinase activity was not significantly increased upon infection by either

M. gisea or *B. glumae* (Figures 7E and 8C). Therefore, the levels of disease resistance appear to correlate with the change of OsMAPK5 kinase activity in rice plants.

8.10 Negative regulation f PR gene expression by *OsMAPK5*

Since *OsMAPK5-RI* lines exhibited elevated resistance to fungal and bacterial pathogens, the expression of some pathogenesis-related (PR) genes in *OsMAPK5-OX* and *OsMAPK5-RI* lines under normal growth conditions. Interestingly, RNA blots showed that two rice *PR* genes, *PR-1b* and *PR-10*, were constitutively expressed in *OsMAPK5-RI* T_1 transgenic seedlings in the absence of pathogen infection, but not in non-transgenic or *OsMAPK5-OX* seedlings grown under the same conditions (Figure 9). Similar results were obtained in T_0 transgenic plants and leaf tissues from different developmental stages (data not shown). These data suggest that OsMAPK5 could negatively modulate (probably through an indirect effect) *PR* gene expression (at least *PR-1* and *PR-10*) as well as broad-spectrum disease resistance.

8.11 Positive regulation of drought, cold and salt tolerance by *OsMAPK5*

The effects of overexpression or suppression of *OsMAPK5* on the tolerance of transgenic plants to cold, drought and salt stresses was examined. Stress tolerance was evaluated based on the percentage of seedlings survived after cold, drought or salt treatment. Surprisingly, the four *OsMAPK5-RI* lines with enhanced disease resistance exhibited significantly ($P<0.001$) reduced tolerance to cold, drought and salt stresses (Figure 10A). In contrast, the three *OsMAPK5-OX* lines showed significantly increased tolerance to salinity ($P<0.005$), drought ($P<0.01$) and cold ($P<0.05$). The kinase activity of OsMAPK5 in transgenic lines was also assayed after the stress treatments. As expected, the normal activation of OsMAPK5 by cold, salinity and drought was suppressed in *OsMAPK5-RI* lines, whereas, the kinase activity in *OsMAPK5-OX* lines was higher than in control plants (Figure 10B). These results suggest that the activation of OsMAPK5 positively regulated plant tolerance to abiotic stresses such as drought, salinity and low temperature.

All references cited herein are incorporated herein by reference in their entirety and for all purposes to the same extent as if each individual publication or patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety for all purposes. Many modifications and variations of this invention can be made without departing from its spirit and scope, as will be apparent to those skilled in the art. The specific embodiments described herein are offered by way of example only, and the invention is to be limited only by the terms of the appended claims, along with the full scope of equivalents to which such claims are entitled.

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